2017-05-19

Characterization of Severe Malaria in Liberian Children 5 Years Old and Younger

Patricia A. McQuilkin
University of Massachusetts Medical School

CHARACTERIZATION OF SEVERE MALARIA IN LIBERIAN CHILDREN 5 YEARS OLD AND YOUNGER

A Masters Thesis Presented

By

Patricia Ann McQuilkin, MD

The signatures of the Master's Thesis Committee signify
Completion and approval as to the style and content of the Thesis

Ann Moormann, PhD, MPH
Chairman of Committee

Jeffrey Bailey, MD, PhD
Member of Committee

Robert Goldberg, PhD
Member of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all master's degree graduation requirements of the school

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

Program
Masters of Science in Clinical Investigation

May 19, 2017

Acknowledgements

I would like to acknowledge the hard work of many of my Liberian colleagues in the work that is presented herein. Benetta Collins Andrews, MD, a Liberian Pediatrician, was the co-investigator on this project. She was responsible for implementing the project in Liberia and overseeing its execution. Dr Shankar, a medical officer in Liberia, helped with study implementation and data input. Eric Adu, the head laboratory technician at JFK MC, took much of his own limited time to help us make this project happen, and oversee the collection of laboratory data and reading of malaria slides. Lucynthia Chea, RN, was the research nurse who did a tremendous job recruiting patients and collecting data. None of the work presented in this mater's thesis would have been possible without this outstanding research team.

I would also like to thank my mentorship team, Ann Moormann, Jeff Bailey and Rob Goldberg for their excellent guidance and patience with this project. Finally I would like to thank Louise Maranda for hours of biostatistical support and teaching.

This work was supported through a pilot project grant from the UMass Office of Global Health.

Abstract

Malaria continues to be a challenging problem in the developing world, and the burden of this life threatening disease continues to be borne by young children living in Sub Saharan Africa. One of the biggest challenges to the prevention and control of this problem lies in accurately diagnosing malaria, and distinguishing it from the many other febrile illnesses which present in children in this age group.

Liberia is a West African country with a high burden of malaria. Very little is known about the presentation of severe malaria in children aged 5 years old and younger in Liberia. We undertook a prospective, hospital -based study of children 5 and under presenting to JKF Medical Center, the national referral hospital, with fever and signs and symptoms consistent with malaria. The aims of our study were to determine: 1) the frequency of confirmed malaria cases, 2) the frequency of non-malaria diagnoses, 3) the prevalence of anti-malarial drug resistance mutations, 4) the presence of other life threatening etiologies of febrile illness such as S. typhii and Dengue virus and 5) immunological profiling associated with severe malaria.

We analyzed clinical and laboratory data from 462 children age 5 and under who presented to the national referral hospital in Monrovia, Liberia with signs and symptoms consistent with malaria over a one year period. Key findings included

determining the demographic factors most closely associated with severe malaria in this population (age > 1yr and urban environment) and those that were negatively associated with the development of severe malaria (prior episodes of malaria, use of bednets and use of anti malarial medications prior to presentation). The clinical symptoms most closely associated with severe malaria in this population were found to be headache and vomiting.

We found that 33% of children admitted and treated for severe malaria did not test positive for malaria by rapid diagnostic testing (RDT) or blood smear. These children had a case fatality rate that was 5 times higher than their RDT positive counter parts. Of the RDT negative children, 2 tested positive for salmonella typhii, but were not treated for this pathogen. Upon discharge from the hospital, 11% of children had resolved their symptoms, but had not cleared their malaria parasites.

These findings will help to identify the children who present with true severe malaria in Liberia. They also underscore the need to expand diagnostic capabilities to determine which other types of pathogens cause febrile illness in this population, so that adequate treatment can be extended to these patients.

The immunoprofiles of these children revealed 3 IgM antibodies (AMA-1, CSP and LSA-1) that were associated with the development of severe malaria. These

antibodies also appear to be associated with initial infection with malaria. Such data will help to identify antigens could be potential targets for malaria vaccines, and which can play an important role in the development of new malaria diagnostics for this population.

Table of Contents

List of Tables and Figures

Chapter I: Introduction

According to the World Malaria Report 2016, there were 214 million cases of malaria globally in 2015 and 438,000 malaria deaths (1). The burden of malaria deaths has been heaviest in the WHO designated African Region, where an estimated 90% of all malaria deaths occurred. Secular trends in death rates, and total malaria deaths, are dominated by West Africa, which accounts for the highest number of all malaria deaths in Africa (31%). The burden of malaria deaths in Sub Saharan Africa (SSA) continues to be shouldered by children less than 5 years old, who account for more than two thirds of all malaria deaths, and the highest number of deaths and highest death rates on the African continent (2).

There are several challenges to accurately diagnosing and treating malaria in SSA. In low resource countries, where there is a high burden of malaria, differentiating malaria from other causes of febrile illness can be challenging. Many of the signs and symptoms of malaria are shared with other infectious diseases that are prevalent in the region. A striking example is the recent Ebola Virus outbreak in Liberia, which posed many diagnostic and management challenges, since Ebola Virus Disease (EVD) shares many of the same symptoms as malaria (3). Other etiologies of febrile illness, such as Salmonella typhi and viral infections, are also prevalent, but severely constrained laboratory

resources make a definitive diagnosis and effective treatment difficult to determine by clinical findings alone.

Many studies have demonstrated that in high malaria transmission areas, a large percentage of the population can carry malaria parasites asymptomatically, without showing signs of the disease (4). Patients develop partially protective immunity against the parasite after repeated infection, and will test positively on malaria diagnostic tests, even though malaria may not be the cause of their acute illness. This can cause confusion when patients present with febrile illness, since these patients can be treated for malaria, while the true cause of their illness may be overlooked. The development of tolerance to the malaria parasite in this setting is thought to occur in young children, as they become repeatedly exposed to the malaria parasite (5). A better understanding of the development of this natural immunity is useful in learning how to develop targets for a malaria vaccine.

Finally, the development of *Plasmodium falciparum* antimalarial drug resistance is one of the major obstacles in the fight against malaria (6). Recent studies combining data sets from the Global Burden of Disease Study (7) and the Malaria Atlas Project (8) demonstrate that various preventive and treatment measures, including the use of insecticide treated bed nets, malaria rapid diagnostic tests (RDT), and treatment using artemisinin-based compounds have

been responsible for a large decrease in the total number of malaria deaths since 2010, and a decrease in the incidence rate of new malaria cases in the African region. However, concern about achieving sustained malaria control and eventual elimination have arisen in light of the recent development of artemisinin resistant parasites in Southeast Asia (9). Timely information on the scope of antimalarial drug resistance is essential in advancing malaria control programs. Using molecular markers it is possible to screen parasites for the gene mutations that convey antimalarial drug resistance. For example, a study in Ghana in 2005 demonstrated the presence of mutations in the *pfcrt* and *pfmdr1* genes associated with chloroquine and possibly with amodiaquine and mefloquine resistance (10).

In order to monitor global malaria control, countrywide programs have been initiated. Liberia is a West African country with a high clinical and public health burden of malaria. Recent data from the President's Malaria Initiative shows that malaria continues to be a significant problem in Liberia, and accounts for more than 40% of outpatient visits and 33% of inpatient deaths (11). All geographic areas are at risk and transmission is perennial. The major parasite species are *Plasmodium falciparum* (>90%), *P. ovale,* and *P. malariae*. The most recent Liberian Demographic and Health Survey performed in 2013 (12) showed a malaria prevalence rate of 28% nationally, with considerable variation by region, ranging from 17% in the south central region (which includes the capital city

Monrovia) to 49% in the southeast region. Based on results from the Liberian Malaria Indicator Surveys in 2011 (13), the prevalence of malaria parasitemia in children under age five by RDT was 66% in 2005, 37% in 2009, and 45% in 2011; the prevalence rate as measured by microscopy was 32% in 2009 and 28% in 2011. The Ebola epidemic that struck West Africa late in 2014 disrupted much of the health care system, and it is hypothesized that this may have led to an even greater incidence of malaria parasite frequency; however, due to disruptions in disease reporting, little recent data exists (11).

Pilot data obtained at the national referral hospital, JFK Medical Center from 2009-2011, suggests that 42% of hospitalizations for children less than 5 years old were for the treatment of malaria. Approximately 20 % of children who were presumed to have malaria did not meet the WHO case definition, raising the possibility that alternative febrile illnesses may have been under diagnosed. Furthermore, an additional 5% of patients did not respond to standardized antimalarial treatment, raising the possibility that drug resistance already exists in Liberia and may contribute to treatment failures (unpublished data). Little is known about the prevalence of anti-malarial drug resistance mutations in malaria parasites found in Liberia.

Overall, little has been published about the clinical presentation of malaria in children in Liberia and etiology of alternative diagnoses. The WHO has devised a

case definition for the diagnosis of severe malaria (14), but this working case definition has not been validated in this setting. Little is also known about the effectiveness of treatment for malaria in Liberians and other potentially life-threatening causes of febrile illness that may exist in this population. Since Liberia is a high intensity transmission region for malaria, it is also an ideal setting to evaluate the development of natural immunity to malaria and how sero-surveys could be used to monitor malaria transmission and malaria control efforts – as well as improve diagnoses.

Study Aims:

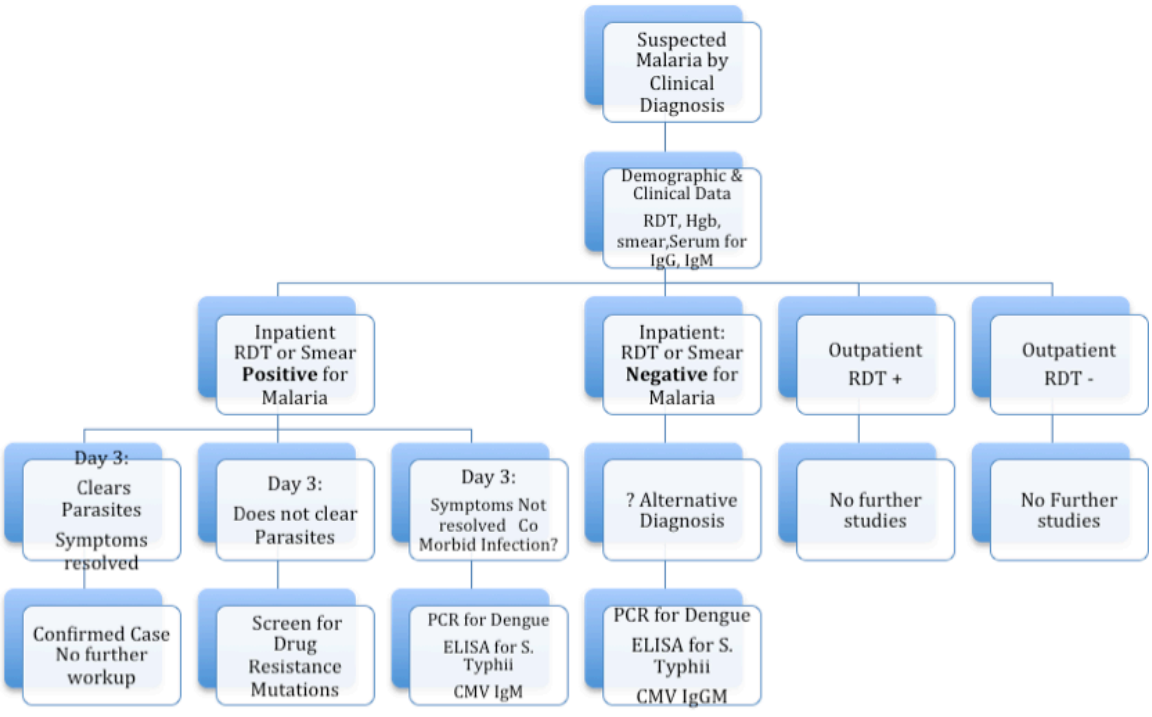
The goals of this prospective, hospital-based, observational study were to provide the following information on children admitted to JFK medical center with *febrile illness*: 1) the frequency of confirmed malaria cases; 2) the frequency of non-malaria diagnoses; 3) the prevalence of anti-malarial drug resistance mutations 4) the presence of other life threatening etiologies of febrile illness such as S. typhii and Dengue virus; and 5) the immunological profiles of children presenting with severe malaria.

Chapter II. Methods

Study overview

We carried out a prospective longitudinal study of the clinical course for children seen for, and/ or admitted with, suspected malaria over a 12-month period from June 2013 through May 2014 at the JFK Medical Center (Figure 2.1). For comparison, we recruited both children being admitted for severe malaria and also outpatients with uncomplicated presentations, so that we could compare patients with severe versus uncomplicated malaria.

Figure 2.1: Study Design



Study setting

The study site, JFK Medical Center, is a tertiary care facility situated in Monrovia, the capital city, and is the National Referral Hospital for the country. Complicated cases of severe malaria are often referred to this hospital for management. The under 5 (U5) unit consists of 30 beds with a census of about 250 admissions per month. Children admitted to this unit are under 5 years of age, and approximately 42% of these admissions are for treatment of malaria. The outpatient clinic sees approximately 1,800 children per month and it is estimated that 40% are seen for suspected malaria.

Patient recruitment

Inclusion criteria for the inpatient study included the following: 1) presentation to the pediatric "U5" unit at JFK Medical Center; 2) signs and symptoms upon admission consistent with severe malaria including prostration, weakness, impaired consciousness, seizures, respiratory distress, and treatment initiated for severe malaria; 3) parental written consent; and 4) age range birth to 5 years of age. Exclusion criteria included children currently on treatment for HIV (since anti-retroviral medication may partially treat malaria) or other complicated illnesses, which may affect immune function, such as cancer or severe malnutrition.

Inclusion criteria for outpatients recruited to the study included the following: 1) presentation to the outpatient clinic with fever and suspected malaria; 2) parental written consent; and 3) age range birth to 5 years of age.

Data Collection

After informed consent was obtained, a research nurse collected demographic and clinical information from an in-person parent interview and chart review (Appendix 1). Blood was obtained for malaria diagnosis, as per usual JFK protocol, and at this time, an additional blood sample was obtained for future research studies.

Children who were hospitalized were also asked to provide additional clinical and laboratory data, either on hospital day 3 or upon discharge, whichever came first, to investigate the clearance of parasites and resolution of presenting symptoms.

All data were transferred to a database that was created in REDCap.

Clinical Assessment

A research nurse who spoke local dialects interviewed parents and obtained demographic information and information about clinical signs and symptoms of malaria. A standardized data collection form was utilized which included WHO criteria of severe malaria. Parents were also asked about other common signs

and symptoms of disease, and were asked about any additional symptoms not listed on the form (Appendix I). The form was used for data collection for both outpatient and inpatient study subjects.

At the time of hospital discharge, or on hospital day 3, whichever was first, the research nurse obtained additional clinical information from inpatient study subjects to assess treatment related outcomes. Blood was obtained for a malaria smear to assess clearance of parasites.

Clinical Laboratory Studies.

In Liberia, the U5 clinical lab was utilized to perform malaria diagnostic lab studies. The *First Response* rapid diagnostic test (RDT), manufactured by Premier Medical Solutions, India, which tests for the malaria HRP2 antigen, was used to diagnose patients with malaria. This test is highly sensitive and specific for diagnosis of *P. falciparum* infection, but will also detect *P. ovale,* so that all known species of malaria in Liberia are detected using this test. Patients with malaria will continue to test positive for 2-4 weeks after initiation of treatment.

Admitted patients also had a malaria smear and hemoglobin level performed, as per standard protocol. The presence of malaria parasites was determined using Giemsa stained slides and read by trained microscopists. Clearance of parasites

was assessed by repeating the malaria slide on hospital discharge or on hospital day three.

Clinical management

The majority of patients were seen initially by a trained medical officer in the outpatient department of JFK Medical Center. Patients who were acutely ill and needed urgent medical attention were brought directly for inpatient admission. Many of these patients would be started presumptively on malaria treatment, due to the acuity of their illness. Outpatients who were found to be RDT positive, but judged by the medical officer providing care to not have symptoms or signs of severe malaria, were treated at home with oral ACT (artemether/lumefantrine), one pill daily for 3 days, according to a standard protocol.

Patients who were judged to have signs and symptoms of severe malaria (RDT positive, symptoms of severe malaria by WHO criteria) were referred for inpatient admission. These patients received a malaria smear and hemoglobin level prior to initiation of treatment. Inpatients were treated using either intravenous quinine or intravenous artusenate. At the time that this study was performed, Liberia national guidelines had recently changed to require intravenous artemisinin based compounds be used for inpatient treatment of severe malaria. However, early in the study, and at some points during the study when stockouts occurred, children were treated with intravenous quinine x 3 IV doses.

After completing the specified course of intravenous treatment, children were discharged home with an additional 3-day course of oral ACT or quinine, depending on which treatment they had received intravenously. Typically, there is no investigation to determine if patients have cleared their parasitemia upon discharge.

Research laboratory methods

Finger prick or venous blood samples for future investigations were obtained from enrolled study participants and collected using sterile technique into BD anti-coagulated Microtainer tubes (~ 500 µl) and also by collecting blood drops onto filter papers. Blood samples obtained on admission for research purposes were centrifuged in the JFK laboratory to separate plasma from blood cell pellet. The samples were then frozen in -20C freezer until transport. Additional blood spots on filter paper were obtained, packaged with desiccant and stored in a similar manner. Samples were transferred by air carrier to UMass Medical School for further investigation.

Molecular techniques: For drug resistance screening, we performed DNA isolation from the pooled filter paper specimens using commercially available DNA extraction kits (Qiagen). PCR primers and restriction enzymes were purchased for the amplification and detection of known malaria drug resistance mutations such as *pfcrt* and *pfmdr1* genes associated with chloroquine and

possibly with amodiaquine and mefloquine resistance, the mutations in the *dhps* and *dhfr* which are associated with sulfadoxine pyrimethamine resistance, and the Kelch 13 mutations which are associated with artemesinin resistance. Methods for performing DNA amplification and isolation were performed using previously published methods (15).

Immunological studies. Plasma was used for serological profiling. We utilized a panel of 8 malaria antigens and antigenic-strain variants using Luminex bead-based suspension assay technology (BioRad). This method enables the detection of a patient antibody titer with the use of a capture antigen conjugated to a fluorescent microsphere, followed by identification using a fluorescently labeled secondary anti-human IgG or IgM antibody. Thus, serum from study participants was analyzed for IgG and IgM anti-malaria antibodies to a panel of malaria antigens including the pre-erythrocyte antigens Liver stage antigen 1 (LSA1) and Circumsporozoite Protein (CSP), the blood stage antigens Apical Membrane Antigen (AMA-1), Histidine Rich Protein 2 (HRP2), Merozoite Surface Protein 1 (MSP-1) 3D7, FUP and FVO allelic variants and the transmission blocking antigen, Cell Traversal Protein (Celtos) (16,17).

Salmonella typhi detection. Plasma samples from patients with severe malaria were also analyzed for the presence of IgM antibodies specific to Salmonella

typhi. This was accomplished using commercially available kits (Corgenix), which were highly sensitive for detection of this pathogen.

Dengue virus detection. Plasma samples from patients who were hospitalized but RDT negative were used to detect presence of Dengue virus NS-1 antigen and IgM antibodies, using techniques described previously (19), and commercially available kits (Corgenix).

Data Analysis

We performed descriptive statistics (means, medians, standard deviations, and 95% confidence limits) on all variables of interest. We explored the relationship among the individual covariates with malaria cases (hospitalized patients who achieved resolution of symptoms and clearance of parasitemia by discharge) and also between cases and non-cases, and patients with severe (patients who were RDT positive, and with symptoms of severe malaria) versus mild disease (patients who were RDT positive, but with no symptoms of severe disease). As appropriate, between group differences were examined using chi-square tests of independence (categorical variables), t-tests (continuous variables) or the equivalent non-parametric tests depending on the distribution of the variables. A two-sided p-value <0.05 was considered to be statistically significant.

We performed logistic regression analysis to determine which demographic clinical characteristics and immunological profiles that were most significantly associated with severe malaria (RDT positive inpatients). Results were age adjusted, since age was noted to be a significant confounder. For those antibodies that demonstrated a large range in mean fluorescent intensity, the levels were broken down into high and low levels, to ascertain whether higher antibody titers were significantly associated with severe malaria onset. Data were analyzed using SPSS.

Ethical Approval

IRB approval was obtained from the Institutional Review Board at JFK Medical Center, Monrovia, Liberia and from the University of Massachusetts Medical School, Worcester, MA, USA.

Chapter III: Results

Patient Enrollment

During the period from June, 2013 to May, 2014, a total of 477 children ranging in age from 0-5 years who presented to JFK Medical Center in Monrovia with fever and suspected malaria were enrolled in this study. Of these, 232 (50.2%) tested positive by RDT for malaria. Of those testing positive, 70.8% met the WHO criteria for severe malaria and were admitted to the hospital for intravenous treatment as previously described. The remaining study participants had uncomplicated infections and were treated on an outpatient basis with oral ACT (Figure 3.1).

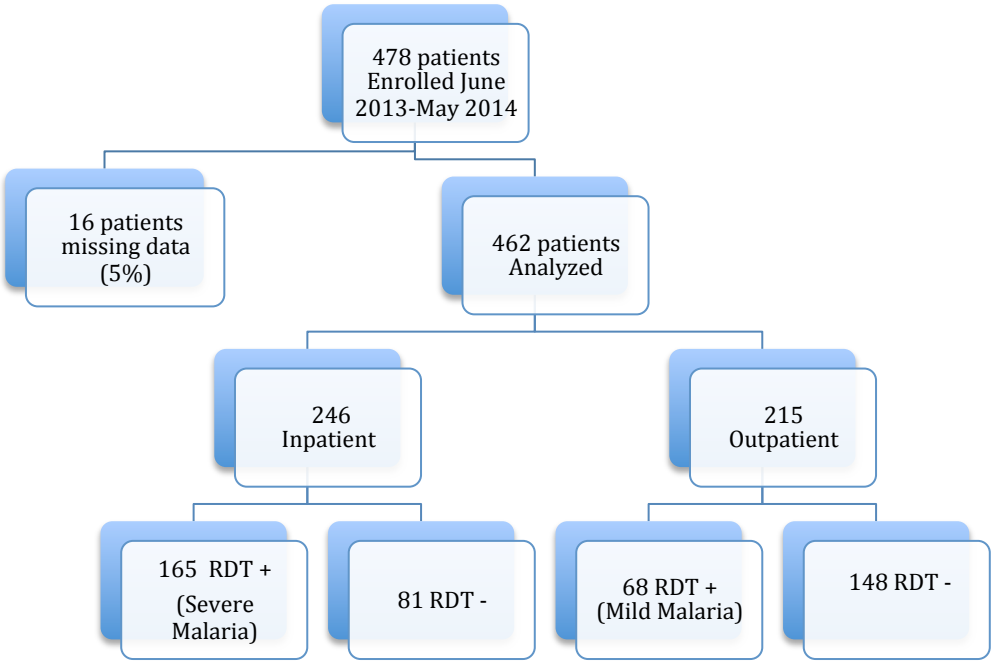


Figure 3.1: Patients enrolled in study

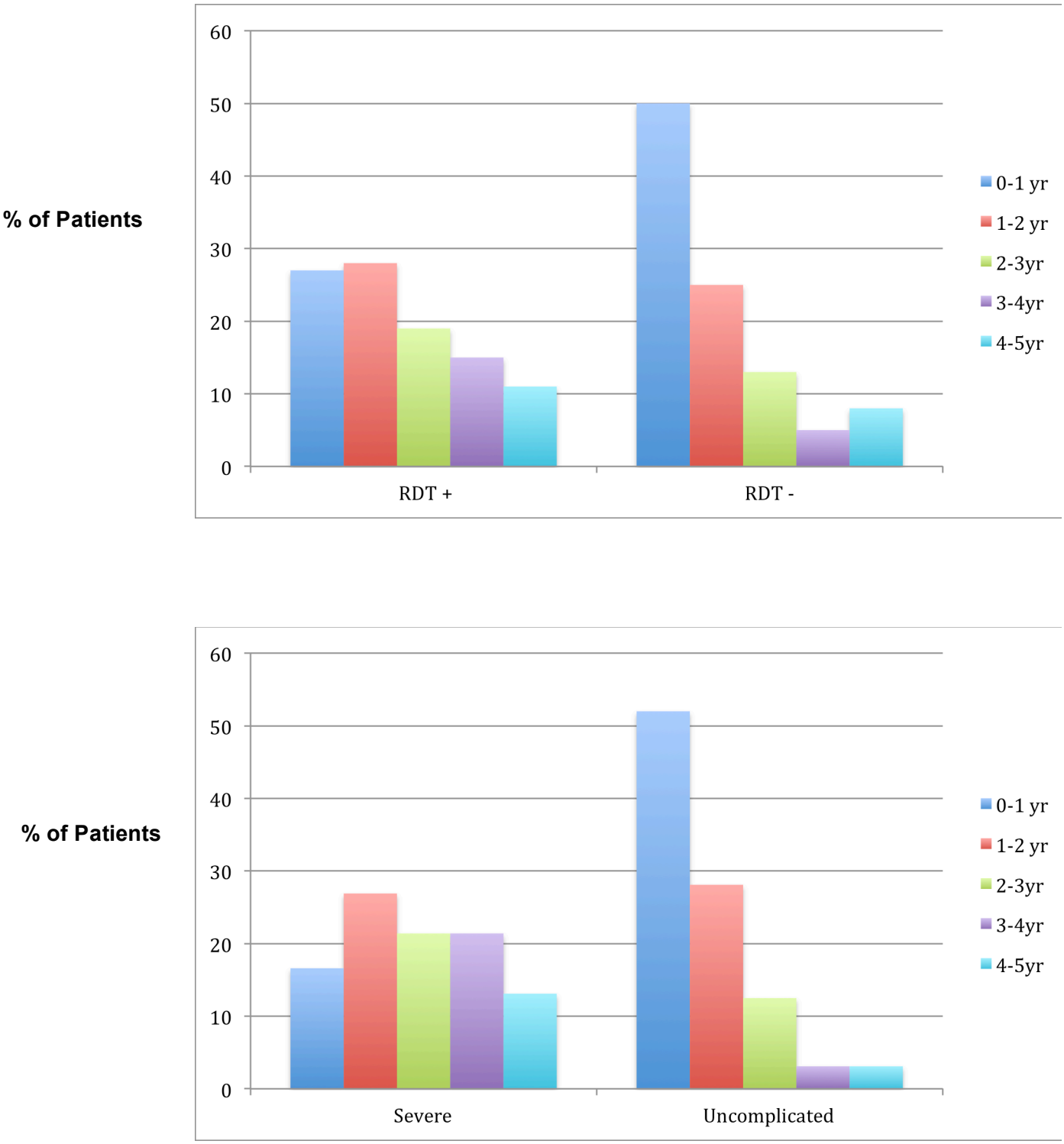


Figure 3.2: Age distribution of respective patient groups . **Upper**: Patients with RDT positive test results versus RDT negative results. **Lower:** RDT positive hospitalized patients (Severe Malaria) versus RDT positive outpatients (uncomplicated malaria)

Table 3.1: Demographic and Clinical Characteristics Based on Rapid Diagnostic Test (RDT) results

Characteristic	**RDT (+)** (n= 232)		**RDT(–)** (n=230)		**P value**
Age (mean, years)	**2.1**		1.4		<0.001
	n	%	n	%	
Female	107	46.1	105	45.7	0.93
Urban	**137**	**59.1**	85	37.0	<0.001
Regular bed net use	50	21.6	**82**	**35.7**	<0.001
Treated for malaria in past	92	40.0	93	40.4	0.92
Anti-malarial use prior to presentation	44	19.1	60	26.1	0.07
Headache	**179**	**77.0**	117	50.9	<0.001
Prostration/ Weakness	**149**	**64.2**	98	42.6	<0.001
Impaired Consciousness	19	8.20	10	4.3	0.12
Pallor	0	0.00	2	0.01	0.24
Seizure	**57**	**24.5**	26	11.3	0.002
Cough	142	61.2	128	55.7	0.26
Diarrhea	50	22.3	51	22.2	0.91
Jaundice	2	0.01	2	0.01	1.00
Respiratory Distress	**38**	**16.4**	17	7.39	0.013
Vomiting	**90**	**38.8**	47	20.4	<0.001
Febrile days (mean)	3.75		3.66		0.68
Hgb (mg/dL) (mean)	9.42		**10.52**		0.04
Parasitemia 0 1-2+ 3-4+ No smear No Data	 10 86 69 9 22		 0 0 0		ND

Demographic characteristics and clinical characteristics of children with malaria.

Demographic characteristics. The average age of patients enrolled in the study was 1.6 years. There were a significantly greater proportion of younger patients less than one year of age in the RDT negative group, 52% vs. 26% respectively. There were also a significantly greater proportion of patients less than one year of age who had uncomplicated versus severe disease, 52% versus 17%, respectively (Figure 3.2).

Less than one half of study patients (46%) were female and 48% of study patients were from Monrovia. Only 31% of parents reported regular bed net use, 40% of children had a history of treatment or hospitalization for malaria in the past, and 60% had no reported prior history of malaria. Overall, 22% had used an antimalarial medication obtained from a clinic or pharmacy prior to presentation to treat the current febrile illness. Of these, 26% were RDT negative patients and 19% were RDT positive patients. Since HRP2 antigen and RDT positivity persists from 2-4 weeks after treatment of active infection, it is unlikely that the RDT negative patients, who typically presented with fever for less than 4 days, were cases of malaria.

Children who tested RDT positive were older and were more likely to be from an urban environment versus those who were RDT negative. Children who tested

negative for malaria were more likely to be sleeping under bed nets regularly as compared with their RDT positive counterparts (Table 3.1, figure 3.3).

Demographic characteristics associated with severe malaria (WHO criteria, inpatient admission) versus those uncomplicated malaria (outpatient treatment) are shown in Table 3.2 and Figure 3.3. Those from urban areas were more likely to present with severe malaria. Alternatively, patients with uncomplicated disease were more likely to have been using bed nets regularly, used an antimalarial medication prior to presentation, and were more likely to have been treated for malaria in the past when compared with those who presented with severe disease.

Clinical characteristics. Clinical signs and symptoms were elicited from all study participants The most common symptoms in this population of children, presenting with febrile illness and suspected of having malaria, were headache (64%), cough (58%), prostration and weakness (53%), vomiting (29%), diarrhea (22%), seizure (18%), respiratory distress (12%), and impaired consciousness (6%) (Table 3.1), In comparing children who were diagnosed with malaria

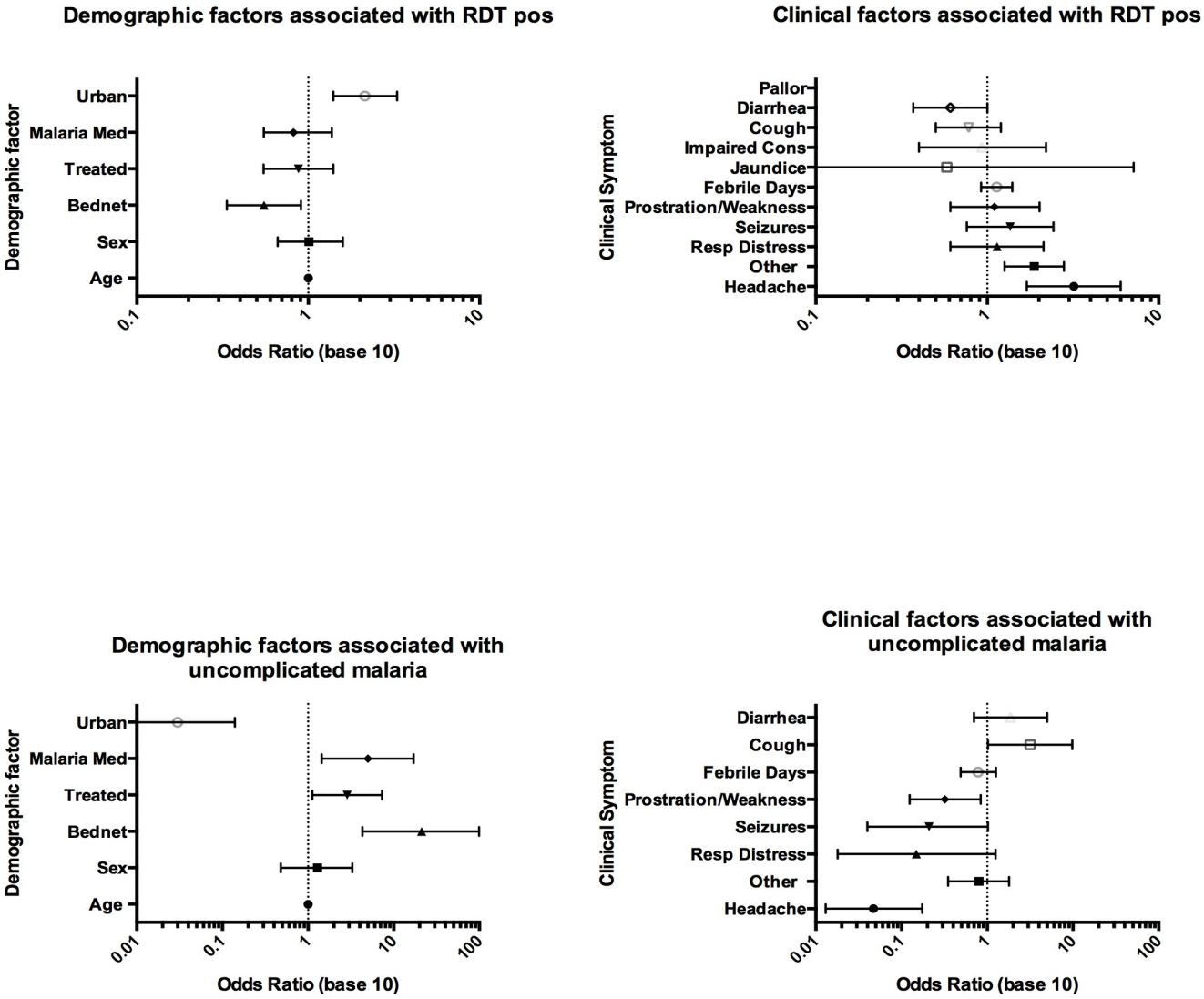


Figure 3.3: Logistic regression analysis of demographic and clinical features associated with RDT positive patients versus RDT neg (top panel) and with RDT positive outpatients (uncomplicated malaria) versus RDT positive inpatients (severe malaria) (bottom panels).

Table 3.2: Demographic and Clinical Characteristics of Patients with Severe (inpatient) Versus Uncomplicated (outpatient) Malaria					
Characteristic	**RDT + Inpatient** (n= 165)		**RDT + Outpatient** (n=68)		**P value**
Age (Mean, years)	2.41		1.37		<0.001
	n	%	n	%	
Female	68	41.2	**39**	**57.3**	<0.001
Urban	**104**	**63.0**	34	50.0	<0.001
Regular bed net use	20	12.1	**30**	**44.1**	<0.001
Treated for malaria in past	48	29.0	**45**	**66.1**	<0.001
Anti-malarial use prior to presentation	8	6.99	**30**	**44.8**	<0.001
Headache	**156**	**94.0**	24	35.3	<0.001
Prostration/ Weakness	**133**	**80.1**	16	23.5	<0.001
Impaired Consciousness	**18**	**10.8**	1	0.00	0.016
Pallor	0	0.00	0	0.00	1.00
Seizure	**54**	**32.5**	3	2.94	<0.001
Cough	107	64.5	35	48.5	0.08
Diarrhea	37	22.3	13	17.6	0.73
Jaundice	1	0.01	0	0.00	1.000
Respiratory Distress	**36**	**21.7**	0	0.01	<0.001
Vomiting	**81**	**48.8**	9	13.2	<0.001
Febrile days (mean)	3.70		3.81		0.67

(RDT positive) versus those who were RDT negative, the clinical symptoms significantly associated with a diagnosis of malaria were headache and vomiting (Figure 3.3). Vomiting was not a symptom on the original checklist; rather it was a symptom that was added when patients were asked about “other symptoms”. It was found to be significantly associated with testing RDT positive in this population. The only clinical symptom significantly associated with being RDT negative was diarrhea.

Clinical data were also analyzed to determine if there were clinical symptoms that were associated with severe malaria (RDT positive inpatients) versus uncomplicated disease (RDT positive outpatients) (Table 3.2). Headache, prostration, weakness and seizures were associated with severe disease. The symptoms most significantly associated with uncomplicated disease included diarrhea and cough (figure 3.3).

Febrile Case Investigations

Patients who were admitted to the hospital with febrile illness and a presumed diagnosis of malaria, and were being treated for malaria, were assessed on admission and followed through discharge or hospital day 3 to determine their outcomes (Figure 3.4).

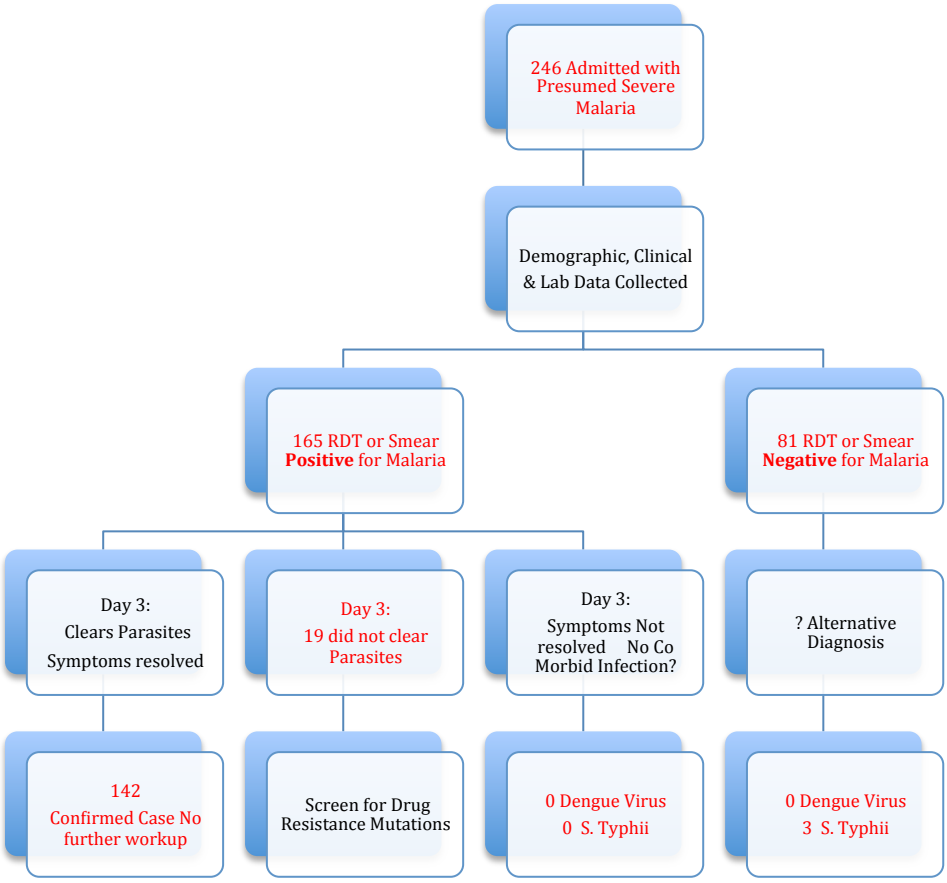


Figure 3.4: Results of febrile case investigations

Among the 246 patients admitted and presumptively treated for malaria, 165 (67%) tested positive for malaria by RDT. There were 5 deaths in this young patient cohort, including 1 in the malaria positive group and 4 in the malaria negative group. The hospital case fatality rate for patients with RDT positive malaria was 1.2%; for those who RDT tested negative, but were still treated for malaria, the case fatality rate was 4.9%.

Of the 165 patients who were admitted and tested positive for malaria, 142 (89%) responded effectively to treatment (resolved symptoms, parasite cleared on day 3 smear). However, 19 patients (11%) admitted with RDT positive malaria did not clear their parasites after treatment and harbored parasite levels ranging from 1+-4+. Nevertheless, the patients in this group had resolved their acute symptoms by day 3 and were discharged home, with malaria positive blood smears (parasitemia is not routinely re-tested after symptoms resolve). The average age of this cohort was 28 months, and only 2 were less than 9 months old. The majority of these patients were treated with intravenous Artusenate as a monotherapy (Table 3.3).

Patients who were hospitalized, but were RDT negative, were screened for two alternative causes of febrile illness, namely Dengue virus and Salmonella typhi. A total of 3 patients (3.7%) who were RDT negative tested positive for Salmonella typhi IgM by ELISA. All of these children, who were older than 3 years, were treated with anti-malarials, and none received antibiotics because this test was done retrospectively as part of this pilot research project. Of the 3 patients, 2 resolved symptoms and were discharged on day. One patient did not resolve symptoms immediately and was hospitalized for 7 days. The RDT negative patients were also tested retrospectively for Dengue virus by ELISA to NS1 antigen, but none tested positive.

*

Table 3.3: Clinical and demographic characteristics of patients who did not clear parasitemia on discharge

Patient	Age In mos.	Sex	Admit Date	Region	Number of malaria infections in past	RDT on Admit	Level of Parasitemia on Admit (0-4+)	Medication Used	Level of Parasitemia On Day 3 (0-4+)
1	52	Male	11/11/13	Montserrado	3	Positive	1+	Artusenate,	4+
2	45	Male	7/24/13	Montserrado	0	Negative	ND *	Quinine	2+
3	37	Female	11/6/13	Montserrado	0	Positive	4+	Artusenate,	4+
4		Male	11/6/13	Montserrado	0	Negative	ND	Artusenate,	1+
5	55	Male	11/7/13	NR**	0	Negative	ND	NR**	1+
6	25	Male	11/7/13	Montserrado	1	Negative	ND	NR	1+
7	38	Male	11/7/13	Montserrado	0	ND	ND	NR	2+
8	37	Female	11/12/13	Bong County	0	Positive	4+	NR	1+
9	17	Male	11/20/13	Lofa	1	Negative	ND	Artusenate,	1+
10	8	Male	12/4/13	Montserrado	0	Positive	2+	NR	1+
11	20	Male	12/18/13	Montserrado	1	Negative	ND	Artusenate,	1+
12	23	Male	1/14/14	Montserrado	0	Positive	1+	Artusenate,	1+
13	1	Male	1/21/14	Montserrado	2	Positive	ND	Artusenate,	1+
14	40	Male	2/23/14	Bomi County	0	Positive	3+	Artusenate,	3+
15		Male	2/24/14	Montserrado	0	Negative	ND	Artusenate	1+
16	19	Male	2/24/14	Grand Cape	0	Positive	1+	Artusenate	1+
17	33	Female	2/28/14	Grand Bassa	2	Negative	ND	Artusenate	1+
18	25	Male	2/28/14	Bong	0	Positive	2+	Artusenate	1+
19	9	Male	4/3/14	Grand Bassa	0	Negative	2+	Artusenate,	1+

*ND- Not done
**NR- Not recorded

Anti-malarial drug resistance

In our original study design, patients who had not cleared their parasitemia by hospital day 3 were to be tested for anti-malarial drug resistance. Unfortunately, due to the onset of the EVD outbreak, we could not transport all of our samples out of Liberia for analysis due to perceived risk and so we were not able to perform drug resistance studies on blood collected from individual patients. However, we were able to pool DNA from a random sampling of 100 RDT positive inpatients, at time of diagnosis, to screen for drug resistance in this population. DNA was extracted from filter paper specimens, pooled and PCR for the four drug resistance mutations was performed. No known drug resistance alleles were found.

Table 3.4: Logistic Regression Analysis IgG Antibody Associated with Severe Malaria (adjusted for age)

IgG Antibody	OR *	95% CI
AMA 1 FVO	3.29 (low titer) 2.76 (high titer)	1.17-9.22 1.16-6.60
Celtos	3.31	0.99-11.1
CSP	2.72	0.32-23.2
HRP11	2.43 (low titer) 5.09 (high titer)	0.86-6.83 1.45-17.8
LSA1	3.42	0.60-19.4
MSP1 3D7	3.33 (low titer) 3.17 (high titer)	0.91-12.3 1.53-6.57
MSP1 FVO	4.39 (low titer) 3.98 (high titer)	1.22-15.8 1.75-9.02
MSP FUP	3.06 (low titer) 4.27 (low titer)	1.06-8.83 1.89-9.63

Table 3.5: Logistic Regression Analysis: IgM antibody associated with severe malaria (adjusted for age)

IgM Antibody	OR*	95% CI
AMA 1	1.61 (low titer) 8.33 (high titer)	0.75-3.45 1.05-65.9
Celtos	1.21	0.22-6.76
CSP	3.14 (low titer) 1.31 (high titer)	1.44-6.87 1.04-1.64
HRP11	1.13 (low titer) 1.43 (high titer)	0.43-2.93 0.27-7.60
LSA1	3.32 (low titer) 1.48 (high titer)	1.09-10.1 0.28-7.77
MSP1 3D7	1.27	0.74-2.18
MSP1 FUP	9.03 (low titer) 0.95 (high titer)	1.16-70.3 0.33-2.72
MSP1 FVO	1.35 (low titer) 0.82 (high titer)	0.52-3.47 0.33-2.02

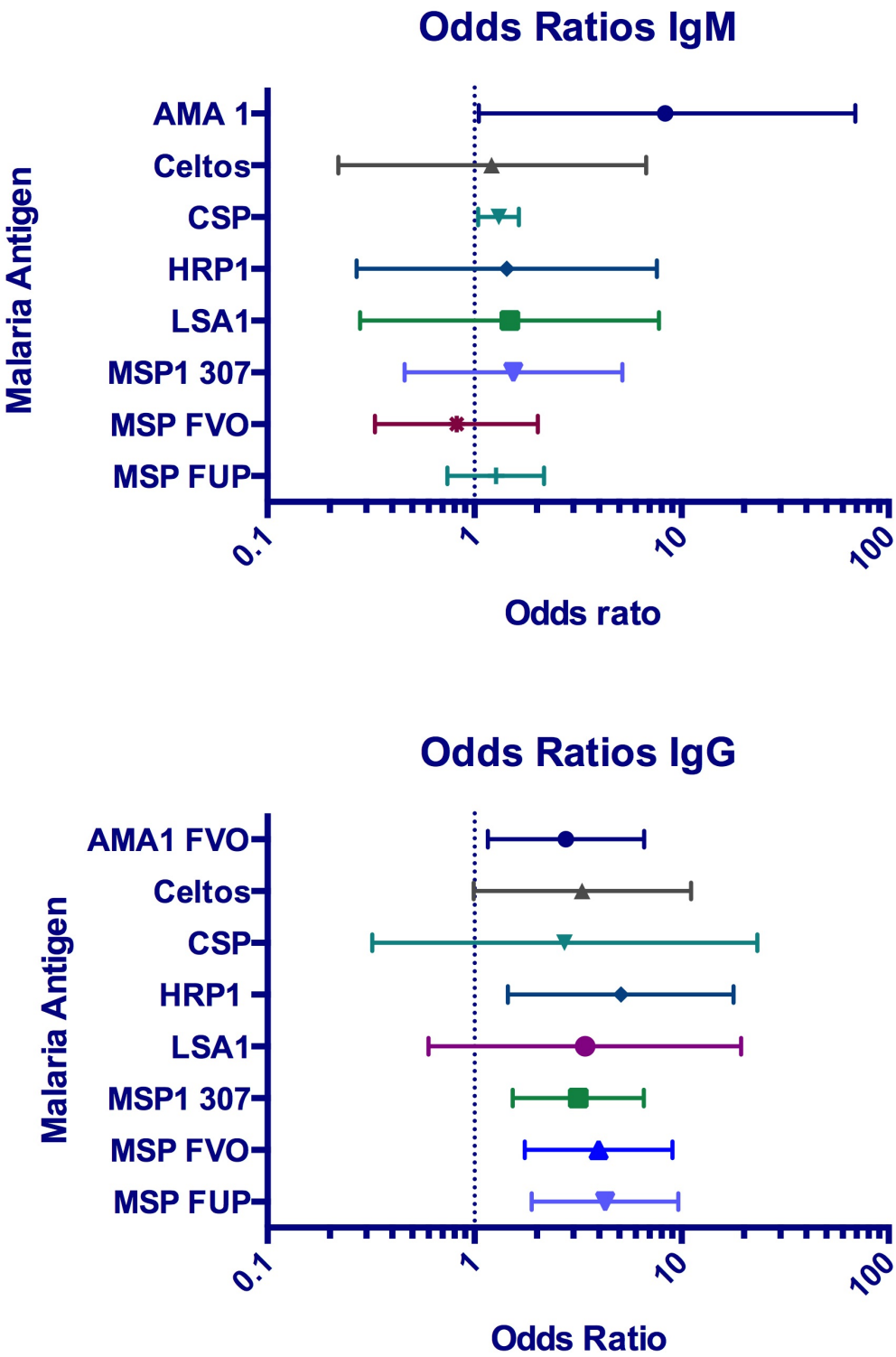


Figure 5: Logistic regression analysis: Odds of antibody being associated with severe malaria, adjusted for age. X axis shows Odds Ratio on logarithmic scale. Y axis shows the malaria antigen associated with the antibody. Upper graph shows odds ratios for IgM, lower graph shows OR for IgG.

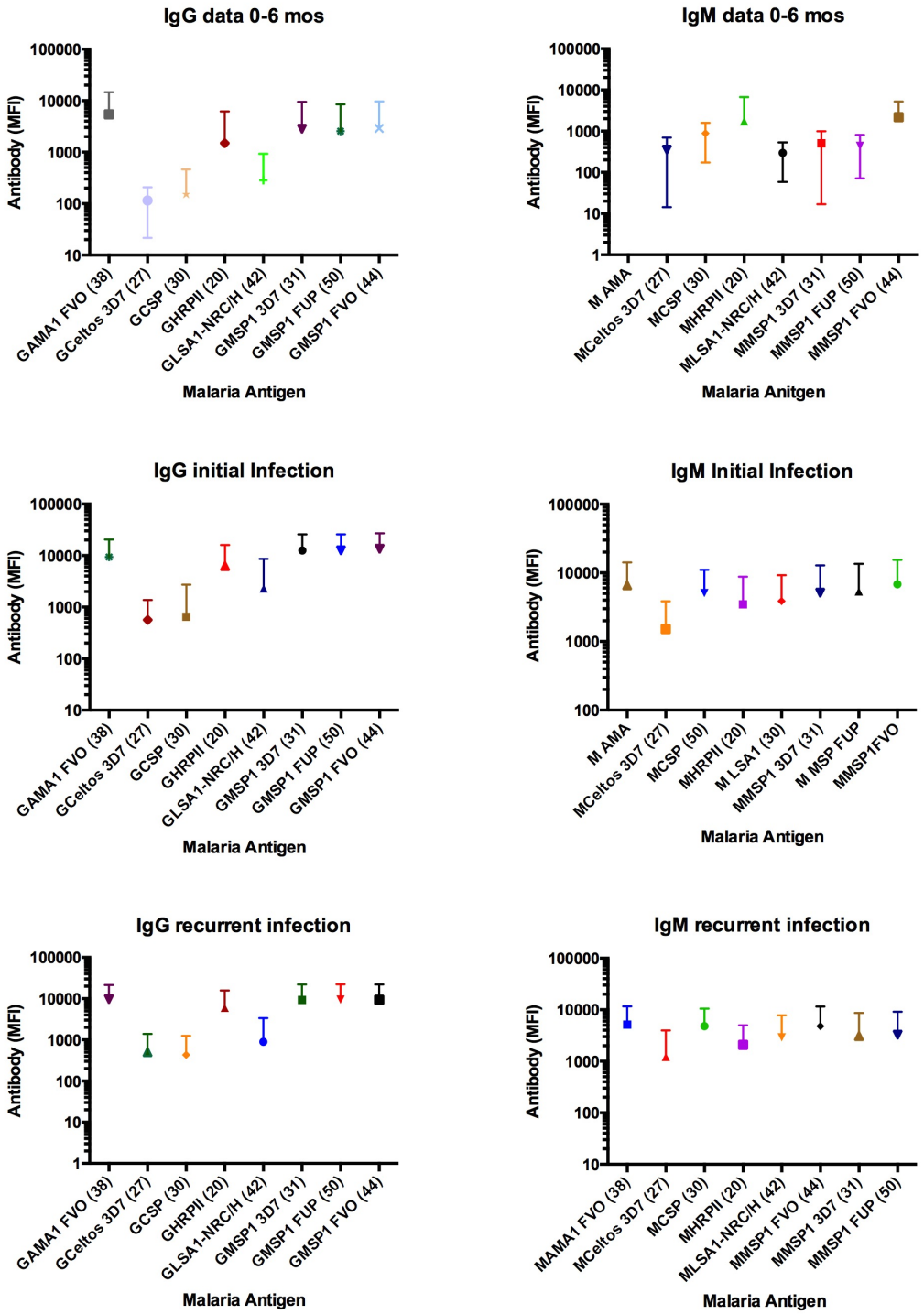


Figure 3.6: IgG and IgM data in uninfected infants 0-6 months of age (maternal antibody) (top panel), in patients with first episode of malaria (middle panel) and in patients with recurrent malaria infection (bottom panel).

Immunological Profiling

Serum samples from all patients enrolled in our study were analyzed using a multiplex immunoassay to determine which anti-malaria antibodies were present in this population.

Our results show that several anti-malarial IgG antibodies were significantly associated with severe malaria in our study population. IgG antibodies to AMA-1, HRP2, Celtos and the MSP-1 allelic variants were elevated in hospitalized patients with malaria (Table 3.3) compared to outpatients. Since these patients were admitted with severe malaria, it is not likely that they were effective in fighting off the infection. IgG antibodies to the pre-erythrocyte antigens, LSA-1 and CSP, were not significantly associated with severe malaria in hospitalized patients (Figure 3.5).

In contrast, IgM antibody levels to LSA-1 and CSP were significantly elevated and associated with severe disease compared to uncomplicated infections (Table 3.4, Figure 3.5). IgM to other malaria antigens were not significantly associated with severe malarial disease.

We examined the presence of IgG and IgM anti-malarial antibodies in infants younger than 6 months of age and with no prior history of infection, in order to identify which maternal antibodies were present in this population. We also

identified which anti-malarial IgG and IgM antibodies were present with initial and recurrent infection, to try to learn more about the development of anti-malarial immunity in this population (Figure 3.7). Antibody associated with initial infection demonstrates which antibodies are responding to an initial challenge with malaria. IgG antibodies present with recurrent infections could be involved with the development of premunition.

Infant less than 6 months of age with no known prior history of malaria by parent report, showed elevated levels of IgG to MSP-1, AMA and HRP2 antigens, but had low levels of LSA1 and CSP IgG. IgM to HRP2 and MSP1 were also noted to be elevated in the 0-6 month old patients. This most likely is an indication that the infants are being bitten by infective mosquitoes but may not become symptomatic because of maternal IgG antibody protection.

In patients with initial infection by parent report, IgM to all malaria antigens that were tested was found to be present. In comparing to figure 3.5, IgM to LSA-1 and CSP (at lower level titer) is significantly associated with severe infection, raising the possibility that these IgM antibodies show potential as diagnostic tool, indicative of severe infection. IgG antibodies

Table 3.6: Correlation Coefficients IgM vs IgG in RDT positive patients (Pearson r, p values)

	IgG AMA-1	**IgG CeLTOs**	**IgG CSP**	**IgG HRP2**	**IgG LSA-1**	**IgG MSP-1 3D7**	**IgG MSP-1 FUP**	**IgG MSP-1 FVO**
IgM AMA-1 (r, p value)	0.04 0.56	0.07 0.29	0.08 0.25	0.05 0.44	0.05 0.50	0.09 0.17	0.09 0.17	0.08 0.21
IgM Celtos (r, p value)	0.05 0.50	0.43 0.53	-0.03 0.70	<0.01 0.91	<-0.01 0.68	0.08 0.27	0.07 0.30	0.07 0.78
IgM CSP (r, p value)	0.06 0.34	0.11 0.10	<0.01 0.96	0.11 0.13	0.10 0.13	0.12 0.07	0.12 0.08	0.11 0.11
IgM HRP2 (r, p value)	-0.02 0.745	0.15 0.02	**0.21** **<0.01*****	0.04 0.55	0.06 0.40	**0.18** **0.01****	**0.17** **0.01***	**0.19** **<0.01****
IgM LSA-1 (r, p value)	0.01 0.07	0.11 0.09	<0.01 0.51	0.01 0.11	0.11 0.30	0.12 0.90	0.12 0.07	0.13 0.69
IgM MSP-1 3D7 (r, p value)	0.02 0.78	0.08 0.24	**0.24** **<0.01*****	0.01 0.85	0.12 0.08	**0.17** **0.01***	**0.15** **0.02***	**0.20** **<0.01****
IgM MSP1-FUP (r, p value)	0.04 0.59	0.11 0.12	**0.25** **<0.01*****	0.03 0.62	**0.14** **<0.01**	**0.21** **<0.01****	**0.20** **0.04***	**0.25** **<0.01*****
IgM MSP-1 FVO (r, p value)	0.11 0.11	**0.16** **0.12***	-0.01 0.92	0.09 0.20	0.12 0.74	0.12 0.08	0.12 0.07	**0.14** **0.04***

Table 3.7: Correlation Coefficients IgM vs IgG in RDT negative patients (Pearson r, p values)								
	IgG AMA-1	**IgG CeLTOs**	**IgG CSP**	**IgG HRP2**	**IgG LSA-1**	**IgG MSP-1 3D7**	**IgG MSP-1 FUP**	**IgG MSP-1 FVO**
IgM AMA-1 (r, p value)	**0.28 <0.01*** **	**0.40 <0.01*** **	**0.35 <0.01*** **	**0.31 <0.01*** **	**0.32 <0.01*** **	**0.45 <0.01*** **	**0.46 <0.01*** **	**0.46 <0.01*** **
IgM Celtos (r, p value)	**0.16 0.02***	**0.19 0.01****	**0.22 <0.01*** **	**0.20 <0.01****	0.067 0.32	**0.24 <0.001*** **	**0.25 <0.001*** **	**0.24 <0.01*** **
IgM CSP (r, p value)	**0.32 <0.01*** **	**0.48 <0.01*** **	**0.22 <0.01*** **	**0.40 <0.01*** **	**0.15 <0.03***	**0.44 <0.01*** **	**0.46 <0.01*** **	**0.46 <0.01*** **
IgM HRP2 (r, p value)	0.01 0.90	0.11 0.11	0.10 0.16	0.04 0.56	0.03 0.68	0.11 0.09	0.11 0.12	0.11 0.12
IgM LSA-1 (r, p value)	**0.28 <0.01*** **	**0.43 <0.01*** **	**0.46 <0.01*** **	**0.39 <0.01*** **	**0.31 <0.01*** **	**0.48 <0.01*** **	**0.48 <0.01*** **	**0.48 <0.01*** **
IgM MSP-1 3D7 (r, p value)	0.069 0.31	0.13 0.05	0.12 0.08	0.063 0.35	**0.26 <0.01*** **	**0.24 <0.01*** **	**0.23 <0.01*** **	**0.25 <0.01*** **
IgM MSP1-FUP (r, p value)	0.11 0.10	**0.16 0.02***	**0.14 0.03***	0.087 0.20	**0.30 <0.01*** **	**0.20 <0.01*** **	**0.28 <0.01*** **	**0.30 <0.01*** **
IgM MSP-1 FVO (r, p value)	0.03 0.70	0.07 0.31	0.08 0.24	-0.09 0.79	**0.14 0.04***	**0.14 0.046**	**0.13 0.05***	**0.16 0.02***

responding to MSP-1, AMA and HRP2 antigens were elevated in these children. In patients with recurrent infection, IgG responding to MSP-1, HRP2 and AMA antigens were again found to be elevated.

We examined the correlations between IgG antibodies and IgM antibodies present in both RDT positive and RDT negative patients (Table 3.6). Patients who were RDT positive, and who had IgM present (indicating initial immune response to a new malaria antigen) had lower numbers of IgG antibodies present

In RDT negative patients, when IgM was present, indicating an initial immune response mounted to a challenge with new malaria antigens, there were a number of IgG antibodies responding to this new infection (Table 3.7). This may represent patients with recurrent infection, who have developed an immune response and can respond with IgG quickly, and thus are fighting off recurrent infection.

Chapter IV. Discussion

In resource-limited settings, such as Liberia, it is presumed that the majority of patients do not seek hospital care, due to distance and expense. In these regions there are few diagnostic resources available to help to distinguish malaria from a myriad of other febrile illnesses. Distinguishing malaria from other causes of febrile illnesses can be useful in identifying those children who need referral for hospital management and alternative treatments other than anti-malarial drugs.

We undertook this pilot study to learn more about how severe malaria presents in this population and whether we could identify factors associated with severe disease in young children 5 and under. To our knowledge, there have been no recent studies that describe the clinical features of severe malaria in Liberian children. We identified several factors that may help identify children who are at risk for severe disease and could be incorporated into a diagnostic algorithm. Our results are consistent with other studies of severe malaria in African children from other countries and may shed light on ways to improve differential diagnoses of acute febrile illnesses.

Clinical and Demographic Features of Severe Malaria in Liberian Children

Consistent with other studies, we found that age is a significant risk factor in this population of children who are exposed to constant, high level of malaria

transmission. In our population, the mean age of children who were RDT positive for malaria was 2.1 years, with children slightly older (mean age 2.4 years) needing to be admitted with symptoms of severe malaria. Very young children, from age 0-12 months, appeared to have less of a risk of testing positive for malaria even when symptomatic. This likely represents the presence of maternal IgG antibodies which confers protection from some infections and is thought to persist from birth up to 6-9 months of age (19). Thus, infants most likely do not have malaria even if the symptoms are presumptive, suggesting that alternate diagnoses should be clinically evaluated. In contrast, slightly older children (1-4 years) were more likely to have severe malaria, and this finding is consistent with waning maternal antibody with persistent exposure to infective mosquitoes (20). These results support a recent malaria indicator survey that was performed in Liberia in 2011, which also concludes that malaria presents in children with varying severity depending on age. (11).

Prior epidemiologic studies of severe malaria have shown that the median age of children affected is inversely proportional to transmission intensity (20). In areas of intense malaria transmission such as Liberia, younger children with undeveloped immunity are more likely to be affected and hospitalized, as was seen in our study. In these regions, malaria is a disease of young children from birth to about 5 years of age, and older children and adults acquire natural immunity after repeated infections (21). In areas with less intense transmission,

older children presenting with impaired consciousness and cerebral malaria may predominate (19). Our findings suggest that in Liberia, young children ages 1-3 years old had the greatest risk of developing severe malaria.

Our finding that only 31% of the population used bed nets regularly is consistent with data from the MIS (11). This survey indicates that while 50% of the population owns a bed net, they do not appear to be using them regularly or correctly, since only 32% of persons surveyed had slept under a bed net regularly. This sample may be biased, however, as this was a hospital based study of children presenting with fever, and not a true community based sample. It does suggest, however that more aggressive malaria control measures are needed in Liberia. Public health measures designed to improve this intervention have proven very successful in other malaria endemic regions of the world (22) and there is no reason to believe they would not become equally successful, if implemented, in Liberia. However, more country-specific studies are warranted to determine if the barriers to bednet use are lack of knowledge of their benefits or other challenges such as access or preference, since sleeping under bednets in hot humid climates makes them uncomfortable to use.

We also found that approximately 22% of patients had been given antimalarial drugs prior to presentation at hospital and this was highly associated with less severe malaria compared to children who had not be given prompt treatment.

Anti-malarial medications are freely available for purchase in pharmacies in Liberia. If accompanied by rapid diagnostic testing prior to administration of anti-malaria medication, pharmacy diagnosis and prompt treatment could prove to be an effective means of preventing cases of severe malaria in children. This is encouraging and demonstrates that parents are knowledgeable about malaria and seek treatment for their children if symptoms do not resolve. Future studies could address delays in care seeking behaviors of parents and determine if RDTs were used by local pharmacies or if the parents presumed their children had malaria without confirmation.

We found being from an urban area was associated with severe malaria. Because the ability to access to care in rural areas is very challenging in Liberia, it is possible that those children with severe disease were unable to reach care in urban areas, where most secondary and tertiary facilities are located.

In comparing patients with severe versus uncomplicated malaria, we found that a higher percentage of patients with uncomplicated presentations had reported prior episodes of malaria. This suggests that children with reported past infections had an opportunity to develop semi-protective antimalarial immunity that would result in uncomplicated infections compared to those with no reported past infections. This finding is consistent with other reports in the literature (20).

One of the primary motivations for this pilot study was to determine how many presumed malaria infections were caused by malaria, as opposed to another co-infection (23,24). The WHO has established criteria for severe malaria, and among these, the most commonly seen symptoms that are predictive of severe disease and death in children include impaired consciousness, respiratory distress, hypoglycemia, acidosis and hyperlactatemia. Other symptoms such as prostration, multiple convulsions, and severe anemia were seen frequently in children, but had lower prognostic value (25). Our findings are consistent with these observations and provided reassurance that clinical signs and symptoms of malaria are informative in this setting. A large number of children in our population also presented with symptoms of headache and vomiting, and this was highly associated with testing positive for malaria and having severe disease. These symptoms have frequently been described in populations presenting with malaria, although are not listed as WHO criteria (26, 27).

A large study performed in Kenya in 1995 (28) sought to validate the WHO criteria for severe malaria in Kenyan children by tracking treatment response and parasite clearance in addition to resolution of symptoms. Their findings suggest that two clinical syndromes of severe malarial disease are present in children: malaria with impaired consciousness, suggestive of cerebral malaria, and malaria with respiratory distress. Both of these symptoms, impaired consciousness and respiratory distress were highly associated with mortality. Cerebral malaria is

more often seen in older children, and respiratory distress, caused by severe malarial anemia, is seen in younger children (28).

Febrile Case Investigations

Many public health efforts designed to address the management of malaria in Africa have concentrated on bringing patients with febrile illness to hospital attention, but few studies have examined the accuracy of diagnosis and appropriateness of hospital treatment. Current protocols in use to diagnose severe malaria are based on reducing the risk of mortality and are sensitive, but not specific (29). Yet the presumptive diagnosis of malaria, and the consequent neglect of alternative diagnoses in hospitalized patients have been demonstrated to impact morbidity and mortality. A recent prospective hospital based study performed in 10 hospitals in Tanzania examined the outcomes for patients admitted and treated for severe malaria. This study concluded that patients admitted with a diagnosis of malaria who were slide negative were often not treated with antibiotics, and had twice the hospital case-fatality rate of their slide positive counterparts (30).

Our results support the observation that children who live in high malaria transmission zones are over diagnosed with malaria. Approximately one third of

the children in our study who were admitted and treated for severe malaria tested RDT negative on admission. Approximately 22% of these children did received an antimalarial medication prior to hospital admission, but since the RDT that is used in this hospital, which tests for HRP2 antigen, is known to stay positive for up to 21 days after diagnosis, it is unlikely that these children were partially treated malaria cases. All were also parasite smear negative. A limitation of this study was that few other laboratory investigations were available to ascertain alternative causes of illness. Consequently, we noted a four-fold increase in case fatality rates in the RDT negative patients versus the RDT positive patients, suggesting that their deaths were caused by an alternative, undiagnosed and therefore untreated illness.

Alternative Causes of Febrile illness

The numerous causes of febrile illness in pediatric populations in limited resource, tropical countries are often difficult to distinguish by clinical findings alone. In African children, febrile illness is the most common reason for hospitalization and malaria and invasive bacterial disease are the most common etiologies (31). Other undiagnosed illnesses can mimic the signs and symptoms of malaria and present in a similar fashion. These diagnoses may not even be considered in children who are admitted and presumed to have severe malaria. For example, recent reports from Malawi, Tanzania and Bangladesh, etc. has shown the incidence of Dengue virus to be increasing (32, 33). Dengue virus

was recently reported in neighboring Sierra Leone (34). S. Typhii is also a common pathogen in African children hospitalized with fever. Patients infected with these pathogens display signs and symptoms similar to severe malaria. More recently, the Ebola Virus Disease (EVD) outbreak in West Africa posed many diagnostic and clinical management challenges, given the common presentation of EVD and malaria. Our study was conducted prior to the recent Ebola epidemic in Liberia. Therefore, we were not in a position to test the impact of such a misdiagnosis on clinical outcomes. However, of the children enrolled in our study, most (66%) were indeed diagnosed with malaria. This underscores the continued public health crisis in Liberia and supports continued efforts aimed at malaria prevention and control.

Yet, other common childhood infections are simmering just under the surface. We screened RDT negative hospitalized patients for S. typhii, a pathogen known to be endemic in this population (35). Salmonella typhii presents in a similar fashion as malaria, with high fever and vomiting. If left untreated, it can lead to intestinal perforation and death. There were 3 children who tested positive for this pathogen, making it the likely cause of their illness. These children were all over the age of 3 years of age, and presented with high fever and vomiting, signs suggestive of both malaria and S. typhii. Unfortunately, our testing was done retrospectively since this test was not available in Liberia. So, none of the children received antibiotics which supports our finding that children who are

presumed to have malaria are at high risk of being undiagnosed and the true cause of their illness left untreated These findings suggest that older children who are acutely ill and RDT negative should also be treated empirically for other etiologies of threatening illness, using broad spectrum antibiotics, until improved diagnostics can be devised. Point of care rapid diagnostics for S.Typhii exist, but have poor sensitivity and specificity, and are not useful in this setting. An improved method of diagnosing this disease would be helpful in this setting (36).

We also performed a pilot test to screen the hospitalized RDT negative children for Dengue virus, but did not find any cases in this small sample.

Future studies to elucidate all causes of febrile illness will be important to pursue in order to improve public health for young children in Liberia. This knowledge will inform the development of further point of care testing and treatment algorithms for children admitted to the hospital or coming to rural clinics for diagnosis of febrile illnesses. Co-infections with malaria also need to be considered with caution and evaluated based on age of the patient since the development of premonition allows the persistence of asymptomatic parasitemias. The introduction of diagnostic antibody testing could be useful in distinguishing premonition versus true symptomatic infection. The use of IgM antibody test, for example, may indicate first malaria infection and would therefore be treated more attentively. The recent Ebola Virus epidemic in West Africa makes the

development of such algorithms even more urgent, since EVD can mimic the signs and symptoms of severe malaria at first, and if not accurately diagnosed, can lead to the spread of disease throughout hospitals (37). A recent study performed in by D'Acremont et al in Tanzania, in an area where malaria prevalence is decreasing, has helped to elucidate many pathogens in this setting, both viral and bacterial, which cause febrile illness in children in this population (39). This approach would be useful in the West African setting, where little is known about the different causes of fever in children.

Clearance of Parasites

The majority of admitted patients (87%) responded to anti-malarial treatment with intravenous quinine or artusenate, resolved their symptoms, and were discharged home by hospital day 3. However, a total of 19 out of 165 admitted patients were noted to have persistent parasitemias on their day 3 blood smear, and had not cleared their parasites at the time of discharge. Patients are not usually checked for persistence of parasitemia on discharge, so this was a new finding. Artemisinin-based compounds are known to have very short half-lives (in minutes), so when therapy is stopped, unless patients are compliant with oral treatment, they will not clear their parasitemia further. The protocol for treatment includes an additional 3-day course of artemisinin-based combination therapy (ACT) post discharge, so it is possible that patients cleared their parasites post

discharge, but there is no follow up data to support this. Patients do not typically follow up for repeat blood smear after their treatment is completed.

There are several reasons why these patients may not have cleared their parasitemia. In sequentially admitted patients who were parasite positive on discharge, it is possible that the anti-malarial drug used to treat these patients was ineffective, due to circumstances such as expired or counterfeit drugs being used. Nosocomial malaria infection has been known to occur in this setting, but unlikely if these children were being treatment for malaria. The development of drug resistance, which we were unable to document, is also a possibility. Lastly, development of immune tolerance to malaria in these children is also a possibility, as it is known that tolerance develops in this age group.

This group of patients who do not clear their parasitemia will be an important group to follow in future studies for many reasons. It is well known that malaria treatment does not always induce sterilizing immunity, and even though a child may no longer suffer the symptoms of malaria or be at risk of death, they can still harbor parasites. These children who carry malaria asymptomatically also serve as a reservoir to infect others. Therefore, determining if they have cleared their infection is of great importance. Furthermore, those children who continue to harbor parasites after treatment is complete could be doing so because they are developing tolerance to malaria parasites. The mechanisms involved in the

development of this tolerance are important in understanding how immunity to malaria develops. THIs correlate of protection could be useful in strategies used in malaria vaccine development.

Drug Resistance Studies

Anti-malarial drug resistance mutations that are associated with medications such as chloroquine, sulfadoxine pyrimethamin (used for intermittent preventative therapy in pregnancy) and other medications have been reported in Africa (39). A recent study performed in Liberia found that although treatment is highly efficacious, selection of molecular markers in reinfections could indicate a decreased sensitivity or tolerance of parasites to the current treatments, and the baseline prevalence of molecular markers should be closely monitored. This was especially true for the *pfmdr* polymorphisms after treatment with artemesinin combination therapy (40). More recently, a recent case report from Equatorial Guinea suggests that artemisinin resistance may be developing in West Africa (41).

A secondary goal of this study was to screen for potential anti-malarial drug resistance in children who do not respond to treatment. We screened 100 inpatient samples for malaria drug resistance for the pfmdr, pfcrt and Kelch drug resistance mutations, but did not detect any of these mutations. Follow up studies of the patients who do not clear their parasitemia on discharge, such as

the 19 patients that we identified, would have allowed us to determine if their parasites had developed drug resistant mutations. However, this pilot study was limited in scope and financing, not to mention that this time frame would have put us at the beginning of the Ebola epidemic.

Immunological Profiling

Natural immunity to malaria develops over time and through repeated infections to variant strains circulating in the population. The development of anti-malarial antibodies is thought to be important in developing this immunity. Most serology studies measure IgG to malaria antigens as indicators of protection and the development of immunologic memory. An antimalarial sero-profile has also been used to indicate a history of past infections and may become a useful tool in malaria control and surveillance programs. However, few studies measure IgM antibodies which would indicate new infections. We examined whether measuring IgG and IgM could be used as a better indicator of new malaria infections that could become integrated into a diagnostic algorithm. This would complement the RDT, especially in children who were given antimalarial treatments prior to coming to the hospital and may still be suffering from their malaria infections even after parasites have been cleared.

Infants are thought to have acquired passive anti-malarial immunity at birth from the placental transfer of IgG antibodies from their mother that have been shown

to persist for the first 6-9 months of life. At the point when this antibody protection starts to wane, children become susceptible to severe disease, as their own immunity is developing (indicated by IgM antibodies). As children age and ((experience repeated)) better descriptor needed, often uncomplicated through symptomatic malaria infections, they start to develop premonition (??), characterized as being less susceptible to severe disease when infected. There is currently no diagnostic test that indicates on an individual basis when this immune tolerance develops and to which malaria antigens this immunity is most directed.

Our results suggest that IgM to AMA-1, LSA-1 and CSP are associated with severe malarial infections. These antibodies may also play a role in initial, severe infections, since in RDT positive patients, they are elevated and do not appear to be correlated with elevations in IgG antibody. These antibodies may be useful in distinguishing which patients may be experiencing initial, severe infection from those who have had malaria in the past, and have developed tolerance.

Our study identified several anti-malaria IgG antibodies that are present in Liberian children who were admitted with severe malaria, such as IgG antibodies to AMA-1, HRP2, Celtos and the MSP-1 allelic variants. These antibodies have been shown to correlate with protection in other populations (42, 43); however,

since they are often found in hospitalized patients, it is unlikely that they conferred adequate protection from developing severe malaria. We found 2 antibodies, IgG responding to LSA1 and CSP, that were not elevated in patients with severe malaria. It is possible that the lack of these antibodies could be associated with lack of protection, and that these particular antibodies confer protection from severe malaria. The patients with severe malaria may not have developed the ability to produce these antibodies.

Many of the children had IgG to blood stage and transmission blocking antigens. Since many of our patients were very young, this IgG may represent passive immunity acquired through maternal antibody. Severe malaria was also significantly associated with IgM antibody responding to two pre-erythrocytic antigens, CSP and LSA -1. It is possible that the addition of antibody titers to these antigens could improve diagnosic capabilities, especially in recently treated children who are determined to be RDT negative.

Study Strengths and Limitations

Strengths of this study include the fact that, to our knowledge, this is the first study investigating the demographic and clinical characteristics of malaria in Liberian children since their second civil war which began in 1999. Our data are also novel in that they were collected prior to the EVD epidemic, and may be helpful in establishing baseline characteristics of this population.

Our pilot study had several limitations that need to be kept in mind in interpreting the study results. We worked with a relatively small sample of patients who were referred to, and treated at, the national referral hospital. This population of patients may not be representative of the Liberian population as a whole, so we do not know if our results are generalizable

Our limited budget prevented full exploration of WHO signs and symptoms of malaria, such as hyperglycemia, acidosis, and hyperparasitemia. These lab investigations are not routinely performed on patients at JFK Medical Center; therefore we were not able to validate symptoms with clinical diagnostics. We were also unable to screen for other types of pathogens known to be prevalent in African children, such as non-typhoid salmonella, and Strep pneumococcus.

Loss of sample integrity and inability to get all of our samples out of Liberia due to the EVD epidemic prevented complete assessment of antibiotic resistance in those children who did not clear their parasitemia

Future Directions

This pilot study points us to several important future studies including the following:

Development of diagnostic and therapeutic algorithms for children presenting with fever and suspected malaria in resource limited settings.

Algorithms which target those at risk for severe malaria, and which provide for treatment of alternative illness in children who test negative for malaria need to be developed. New diagnostics may help to further differentiate those children who are at highest risk.

Elucidation of the alternative causes of acute febrile illness providing better understanding of full spectrum of febrile illness in Liberian children. As we demonstrated, there is a higher case -fatality rate in hospitalized children who test negative for malaria. A better understanding of alternative causes of illness will help improve the overall outcomes for children under 5 years – a Millennium Goal for the WHO. Given the fact that Ebola is now endemic in West Africa, the development of algorithms and clinical guidelines to immediately differentiate the many causes of febrile illness is critical.

Investigation of the development of immune tolerance to malaria in young children. A follow up cohort study to better understand the interplay of passive immunity in neonates, and the development of natural immunity in young children in areas of high malaria transmission will help to identify potential antigenic targets for malaria vaccines.

Investigation of drug resistance A study targeting children who do not clear their parasites after treatment would be useful to screen for drug resistance mutations, and to better understand the mechanisms involved in the development of drug resistance in young children.

Appendix I: Data Collection Sheet

Malaria Study: Data Collection Sheet

Study ID #:

Demographic information: Date of admission:_____

MR #_____DOB_____ Sex: Male_____Female_____

County:_____ % of time bednet used _____ Not used_____

Pre Hospital information:

Number of times treated for malaria _____Number of times in hospital_____

Type of malaria medication Used Prior to this Admission (for this illness):

Number of days with fever prior to admission: _____

Other illnesses_____

Symptoms/Signs on admission:

Headache_____ Prostration/weakness_____ Loss of consciousness_____

Pallor_____ Seizures_____ Cough_____

Respiratory Distress_____Diarrhea_____Jaundice_____

Labs on admission:

Rapid Diagnostic Test: ____positive____negative___Not done

Level of Parasitemia by smear_: 1+___ 2+___ 3+ ___ 4+ ___

Blood glucose_____mg/dl Hemoglobin level_____mg/dl Spot test:_____pos___neg

Discharge Information:

Level of Parasitemia by smear: 1+___ 2+___ 3+___ 4+___

Other diagnoses in addition to malaria:

_____

Antimalarial Medication used _____ Antibiotics used_____

Blood transfusion required?: Yes_____No____

Number of days until clinical status improved_____Number of days in hospital_____

Disposition: Home _____feeding center _____ expired _____other hospital _____

Chapter V: References:

1) WHO World Malaria report 2016
2) Gething, PE,Casey, DC, Weiss D. et al Mapping Plasmodium Falciparium Mortality in Africa between 1990-2015 *New England Journal of Medicine* 375:25 (2016)2 435-45.
3) "Ebola Virus Disease in West Africa — The First 9 Months of the Epidemic and Forward Projections." *New England Journal of Medicine* 371.16 (2014): 1481-495. Print.
4) Dauberies P, et al (1996) Rapid turnover of Plasmodium falciparum populations in asymptomatic individuals living in a high transmission area. Am J Trop Med Hyg 54:18-26.
5) Farnert, Al, Rooth I, Svensson, Snounou G, Bjorkman A (1999) Complexity of Plasmodium falciparum infections is consistent over time and protects against clinical disease in Tanzanian Children. J Infect Des 179:989-995.
6) The World Health Organization Global Report on antimalarial drug efficacy and drug resistance. WHO, Geneva, 2010.
7) Reddy, K. Srinath. "Global Burden of Disease Study 2015 Provides GPS for Global Health 2030." *The Lancet* 388.10053 (2016): 1448-449. Print.
8) Hay, SI, Snow, RW . THe Malaria Atlas Project: Developing Global Maps of Malaria Risk. Plos Medicine PLoS Med (2006) 3(12): e473. doi:10.1371/journal.pmed.0030473
9) Shannon Takala-Harrison Christopher G. Jacob Cesar Arze Michael P. Cummings Joana C. Silva Arjen M. Dondorp Mark M. Fukuda Tran Tinh Hien Mayfong Mayxay Harald Noedl . Independent Emergence of Artemisinin Resistance Mutations Among *Plasmodium falciparum* in Southeast Asia. J Infect Dis (2015) 211 (5): 670-679.

10)F. P. Mockenhaupt, S. Ehrhardt, T. A. Eggelte, P. Agana-Nsiire, K. Stollberg, A. Mathieu Chloroquine-treatment failure in northern Ghana: roles of *pfcrt* T76 and *pfmdr1* Y86. Ann of trop med. and parasitology (2005) 99 (8): 723-32.

11)The President's Malaria Initiative, Malaria Operational Plan, Liberia, 2016

12)Liberia Demographic and Health Survey 2007. Ministry of Health and Social Welfare, Liberia

13)National Malaria Control Program, Malaria Indicator Survey 2011, Ministry of Health and Social Welfare, Liberia.

14)Warrell, D. A., N. J. White, S. Looareesuwan, R. E. Phillips, M. J. Warrell, D. Bunnag, and K. T. Harinasuta. "The Treatment of Severe Falciparum Malaria." *Bmj* 291.6508 (1985): 1573.

15)Christopher V. Plowe Joseph F. Cortese Abdoulaye Djimde Okey C. Nwanyanwu William M. Watkins Peter A. Winstanley Jose G. Estrada Franco Rene E. Mollinedo Juan Carlos Avila Jose Luis Cespedes. Mutations in *Plasmodium falciparum* Dihydrofolate Reductase and Dihydropteroate Synthase and Epidemiologic Patterns of Pyrimethamine-Sulfadoxine Use and Resistance. J Infect Dis (1997) 176 (6): 1590-1596.

16) Richards, Jack S., and James G. Beeson. "The Future for Blood-stage Vaccines against Malaria." *Immunology and Cell Biology* 87.5 (2009): 377-90.

17)Ballou, W.r., K.e. Kester, and D.g. Heppner. "Pre-Erythrocytic Malaria Vaccines to Prevent *Plasmodium Falciparum* Malaria." *Chemical Immunology and Allergy Malaria Immunology* (2002): 253-61.

18)Emmerich, Petra, Angela Mika, and Herbert Schmitz. "Detection of Serotype-Specific Antibodies to the Four Dengue Viruses Using an Immune Complex Binding (ICB) ELISA." *PLoS Neglected Tropical Diseases* 7.12 (2013).

19) Dobbs, Katherine R., and Arlene E. Dent. "Plasmodium Malaria and

Antimalarial Antibodies in the First Year of Life." *Parasitology* 143.02 (2016): 129-38.

20) Reyburn, Hugh. "Association of Transmission Intensity and Age With Clinical Manifestations and Case Fatality of Severe Plasmodium Falciparum Malaria." *Jama* 293.12 (2005): 1461

21) Doolan, D. L., C. Dobano, and J. K. Baird. "Acquired Immunity to Malaria." *Clinical Microbiology Reviews* 22.1 (2009): 13-36.

22) Zamawe, Collins O. F., Kanan Nakamura, Akira Shibanuma, and Masamine Jimba. "The Effectiveness of a Nationwide Universal Coverage Campaign of Insecticide-treated Bed Nets on Childhood Malaria in Malawi." *Malaria Journal* 15.1 (2016).

23) Choge, Joseph K., Ng'Wena G. Magak, Willis Akhwale, Julius Koech, Moses M. Ngeiywa, Elijah Oyoo-Okoth, Fabian Esamai, Odipo Osano, Christopher Khayeka-Wandabwa, and Eliningaya J. Kweka. "Symptomatic Malaria Diagnosis Overestimate Malaria Prevalence, but Underestimate Anaemia Burdens in Children: Results of a Follow up Study in Kenya." *BMC Public Health* 14.1 (2014).

24) Reyburn H. et al (2004) Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study. BMJ.

25) World Health Organization. Severe falciparum malaria. Trans R Soc Trop Med Hyg. 2000;94(Suppl 1):S1–S90.

26) Wiwanitkit, V. Headache and Malaria: A Brief Review . Acta Neuro. Taiwan 2009; 18:56-59

27) Jackson, LC, Malaria in Liberian children and mothers: Biocultural perceptions of illness and clinical evidence of disease.Soc. Sci and Med 20(12):1281-87.

28) Marsh, Kevin, Dayo Forster, Catherine Waruiru, Isiah Mwangi, Maria Winstanley, Victoria Marsh, Charles Newton, Peter Winstanley, Peter Warn, Norbert Peshu, Geoffrey Pasvol, and Robert Snow. "Indicators of

Life-Threatening Malaria in African Children." *New England Journal of Medicine* 332.21 (1995): 1399-404.

29) Bejon P, et al (2007) Defining Severe Falciparum Malaria or Intervention Studies. PLoS Med 4(8):3251

30) Reyburn H. et al (2004) Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study. BMJ.

31) The World Health Organization Global Report on antimalarial drug efficacy and drug resistance. WHO, Geneva, 2010.

32) Faruque, LI, et al (2012). Hospital-based prevalence of malaria and dengue in febrile patients in Bangladesh. Am J. Trop Med Hyg. 2012; 86(1): 58-64

33) Senn, N. et al (2011) Contribution of Dengue fever to the burden of acute febrile illness in Papua New Guinea: An age specific prospective study. Am J Trop Med Hyg 85(1): 132-7

34) Schoepp, RA, Rossi, CA, Khan, SH, Goba, AG, Fair, JN. Undiagnosed acute viral illnesses, Sierra Leone, Emerg Infect Dis. 2014 Jul; 20(7): 1176–1182.

35) Mweu, Evanson, and Mike English. "Typhoid Fever in Children in Africa." *Tropical Medicine & International Health* 13.4 (2008): 532-40.

36) Olsen, S, et al (2004) Evaluation of Rapid Diagnostic Tests for Typhoid Fever. J. CLin. Micro 42(5): 1885

37) WHO Ebola response team, Ebola Virus Disease in West Africa — The First 9 Months of the Epidemic and Forward Projections.N Engl J Med 2014; 371:1481-1495

38) D'acremont, Valérie, Mary Kilowoko, Esther Kyungu, Sister Philipina, Willy Sangu, Judith Kahama-Maro, Christian Lengeler, Pascal Cherpillod, Laurent Kaiser, and Blaise Genton. "Beyond Malaria — Causes of Fever in Outpatient Tanzanian Children." *New England Journal of Medicine* 370.9 (2014): 809-17.

39) The World Health Organization Global Report on antimalarial drug efficacy

and drug resistance. WHO, Geneva, 2010.

40) Otienoburu, Sabina Dahlström, Oumou Maïga-Ascofaré, Birgit Schramm, Vincent Jullien, Joel J. Jones, Yah M. Zolia, Pascal Houzé, Elizabeth A. Ashley, Jean-René Kiechel, Philippe J. Guérin, Jacques Le Bras, and Sandrine Houzé. "Selection of Plasmodium Falciparum Pfcrt and Pfmdr1 Polymorphisms after Treatment with Artusenate–amodiaquine Fixed Dose Combination or Artemether–lumefantrine in Liberia." *Malaria Journal* 15.1 (2016).

41) Lu, Feng, Indigenous Artemisinin-Resistant *Plasmodium falciparum* Emergence in Africa New England Journal of Medicine 2017; 376:991-993.

42) John, CC, Moormann, AM, Pregibon, CC, Sumba, PO, et al. Correlation of high levels of antibodies to multiple pre-erythrocytic plasmodium falciparum antigens and protections from infection. The American Journal of Tropical Medicine and Hygiene, 73(1,) Jul 2005, p. 222 – 228.

43) Chandy C. John Aaron J. Tande Ann M. Moormann Peter O. Sumba David E. Lanar Xinan M. Min James W. Kazura, Antibodies to Pre-erythrocytic *Plasmodium falciparum* Antigens and Risk of Clinical Malaria in Kenyan Children. J Infect Dis (2008) 197 (4): 519-526.